comparatively unstable with an average half-life on the order of only 5 minutes (range 3-8 minutes). In contrast, bacterial rRNA is normally a stable molecule with negligible turnover. There are also examples of some unusually stable bacterial mRNA species with half-lives of an hour or more. Yeast mRNAs are a little more stable than bacterial mRNAs with half-lives on the order of 20 minutes whereas many mammalian mRNAs have half-lives that are much longer on the order of 10 hrs or more. In eukaryotic cells mRNA processing intermediates such as intron species or sequences downstream of poly A sites are relatively short lived with half-lives on the order of 20 minutes or less. In contrast, eukaryotic rRNAs have half-lives on the order of days. **Information on the stability of RNA in cells is common knowledge in the field and does not constitute a new concept.** There have been newer approaches to studying mRNA decay rates more systematically via DNA chips which allows many individual mRNAs to be studied at the same time. This approach has only added to previous studies that dealt with populations of mRNAs.

The present invention demonstrates the incorporation of thio-phosphate into RNA. This results in RNA molecules with nuclease resistant backbones that increase the stability of modified RNAs both inside the cell and outside the cell during extraction procedures. In the parent application US Patent 7,125,982 Figure 2 it was shown that under isolation conditions that normally degrade RNA (bulk is rRNA) to the bottom of a gel, high molecular weight rRNA was detected from cells grown in media containing thio-phosphate. This clearly demonstrates the utility for using in vivo chemically modified RNA to protect RNA during isolation procedures from degradation. Furthermore, in the present invention Figure 1, trace amounts of high molecular weight processing intermediates (hnRNA) of the yeast actin gene were actually detected without any special effort. These species are not normally seen via Northern blotting procedures which measure steady-state populations of mRNAs. Processing intermediates do not normally accumulate because they are rapidly degraded and do not lead to mature mRNA species. However, even hnRNA species can accumulate to some degree when stabilized via chemical modification of their RNA backbones in vivo. This demonstrates the potential utility of the method for assisting with the isolation and identification of unusually unstable mRNA species. Note chemical modification of RNA oligos with phosphorothioate is known to protect such molecules from degradation by serum nucleases as well from degradation by nucleases inside cells. This concept alone supports the idea of using the method to protect RNA during isolation procedures. See Matzura and Eckstein (1968) cited in the specification.

Generally rRNA represents the bulk of total RNA in cells and accounts for 80-90% of the total RNA. For Northern analyses one typically needs to add at least 10 ug of total RNA on a gel to see a particular mRNA species. The present invention clearly demonstrates in Figure 1. the ability to detect the yeast actin gene using just ~0.5-1 ug of total RNA when the cellular RNAs were stabilized in vivo using thio-phosphate in the culture media. This demonstrates the effective accumulation of an mRNA species in vivo using the described invention. The actin mRNA accumulates to a high degree because it is preferentially stabilized relative to rRNAs. While thio-phosphate is incorporated into rRNA it is incorporated predominantly as cells grow and make more rRNA for new daughter cells. It is not incorporated because rRNA gets degraded in the cell and more must be synthesized. Therefore one does not expect that adding thio-phosphate will result in much further accumulation of rRNAs inside cells. In contrast, yeast mRNAs do get degraded and they can be stabilized to increase the amount of mRNA present in a cell. Total mRNA typically accounts for 1-2% of total cellular RNA. A ten fold increase in total mRNA would only increase the total mass of RNA in the cell from 1% to 10%. Confluent cultures of yeast can be obtained by overnight growth after a 1 to 20 dilution. For an mRNA with a half-life of 20 minutes this would allow ample opportunity to accumulate since after 3 half-lives most of the mRNA would be degraded. It is not to difficult to see that an mRNA species with a half-life of only 3 minutes would accumulate to a greater extent than an mRNA with a half-life of 20 minutes or even an unusually stable mRNA of 12-14 hrs such as yeast amylase. Since unstable mRNAs are more difficult to isolate and detect, and the present invention offers a clear advantage for allowing the accumulation of such species. Unstable mRNAs are typically rarer owing to their instability and so the method may be of benefit for these species.

Claim 9 deals with the incorporation of thio-phosphate into a complex organism. The parent application demonstrated the incorporation of thio-phosphate into gold fish (figure 3) by simply adding thio-phosphate into the media at a concentration that was higher than the phosphate levels in the normal feed (referenced in present application). The amount of phosphate present in the feed was measured using a color-metric Hanna phosphate test kit. This was found to be around 1 mg/L and so an excess of thio-phosphate was added at

250 mg/L. 25 mg/L was not as effectively incorporated into intestinal DNA. As thio-phosphate is incorporated into both DNA and RNA it is anticipated that one would similarly be able to modify the RNA of organism treated similarly. The present application mentions the use of the Hanna phosphate test kit as well as methods for depleting phosphate from media or making up minimal media using only thio-phosphate as a source of phosphate.

The present invention acts in a global manner stabilizing populations of mRNAs in cells. By design the present invention is not promoter specific like many inducers used to regulate transcription nor is it gene specific such as might be found in a sequenced modified DNA expression construct. When cells are exposed to media that is fully substituted with thio-phosphate it is possible to obtain maximum levels of incorporation into the backbone of RNA. However, this is not the optimum level for enhancing protein expression. The cells grow a bit slower in fully substituted media and total protein synthesis can be reduced as such (see specification). Note that viability is not affected. While every cell type has a specific requirement for phosphate, it is not the absolute amount of thio-phosphate that is important but rather the ratio of thio-phosphate to normal phosphate. The ratio impacts the extent of modification of in vivo mRNAs and their subsequent stability. The ratio is easily controlled as thio-phosphate competes readily with normal phosphate for incorporation. The complete picture is probably a bit more complicated as mRNAs for proteins involved in protein synthesis need to be present in sufficient quantities for protein expression to occur. In this regard, some cyclic effects were observed for bacterial alkaline phosphatase expression in yeast at different ratios The lowest concentration allowable that enhances the desired protein expression, however, is clearly preferred.

The present invention demonstrates that it is possible to manipulate the stability of mRNA in vivo to enhance the protein synthesis of particular gene products. Micro-organisms are often used to generate various products including those from heterologous recombinant DNA expression constructs. Such constructs tend to produce mRNAs that are less stable owing to their being an introduced rather than a natural endogenous mRNA species. The present invention demonstrates using yeast an enhancement of protein expression for a recombinant DNA expression vector involving the of bacterial alkaline phosphatase (AP) gene under the control of an ADH2 promoter which is regulated by glucose repression. As the yeast cells exhaust the glucose in the YPD media the AP gene is actively transcribe. A ten fold increase in protein encoded with this construct was detected using only a 10% ratio of thio-phosphate to regular phosphate. A ten fold increase in total soluble protein expression however, was not observed (see specification). This suggests that some mRNAs are preferentially enhanced at the expense of others. Nucleolytic enzymes secreted by yeast are known to possess relatively more stable mRNAs with half-lives of 10 hrs or more. The present application shows that such enzymes in yeast (lipases and nucleases) were at best slightly induced. The mRNAs for these genes are presumably more stable than the AP construct and do not benefit as much in terms of protein expression by stabilization of their mRNAs in vivo. This example demonstrates as expected that less stable mRNAs can be preferentially stabilized relative to more stable mRNAs resulting in enhanced protein expression of desired protein products. A different ratio of thio-phosphate to normal phosphate resulted in enhanced expression of a recombinant AP gene in bacteria. Under similar conditions using a recombinant beta-galactosidase construct the expression for this gene was reduced. Again even though total soluble protein synthesis in bacteria was not enhanced, examples of one gene product being preferentially stabilized over another was observed in bacteria using thio-phosphate substituted media.

The optimal ratio of thio-phosphate to phosphate to be used in any given system is one that must be experimentally determined. The ratio can vary as a function of the organism, physiological state, media, etc. An example is given where a recombinant beta-galactosidase construct expressed under conditions that induce the gene (lactose containing media), expression of the recombinant enzyme is inhibited using thio-phosphate. The inhibition is maximal at a 30% ratio of thio-phosphate to phosphate and is relived somewhat at higher concentrations. In contrast, when the same recombinant vector is expressed in media containing only glucose (non-inducing conditions) the expression of the enzyme can be enhanced using thio-phopshate.

It is fairly obvious that one must also consider when a gene product is expressed in order to determine when to add thio-phosphate. Whenever protein products are expressed using an inducer added to the media one has to determine the time point at which the protein accumulates to its highest levels. This is necessarily after the gene has been turned on by the inducer and the mRNA has been translated. How long this takes can vary depending on the organism and system. Similarly one can consider determining the optimal time point to add